Epimorphin mediates mammary luminal morphogenesis through control of C/EBPβ

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Abstract

We have previously shown that epimorphin, a protein expressed on the surface of myoepithelial and fibroblast cells of the mammary gland, acts as a multifunctional morphogen of mammary epithelial cells. Here, we present the molecular mechanism by which epimorphin mediates luminal morphogenesis. Treatment of cells with epimorphin to induce lumen formation greatly increases the overall expression of transcription factor CCAAT/enhancer binding protein β (C/EBPβ) and alters the relative expression of its two principal isoforms, LIP and LAP. These alterations were shown to be essential for the morphogenetic activities, as constitutive expression of LIP was sufficient to produce lumen formation, while constitutive expression of LAP blocked epimorphin-mediated luminal morphogenesis. Furthermore, in a transgenic mouse model in which epimorphin expression was expressed in an apolar fashion on the surface of mammary epithelial cells, we found increased expression of C/EBPβ, increased relative expression of LIP to LAP, and enlarged ductal lumina. Together, our studies demonstrate a role for epimorphin in luminal morphogenesis through control of C/EBPβ expression.

Introduction

The majority of mammary gland development occurs postnatally. During puberty, a system of branching ducts penetrates the fatty stroma; in pregnancy, the epithelium continues to proliferate, developing additional complexity and lobulo-alveolar structures. Many of these developmental processes are dependent upon reciprocal communication between the stroma and the epithelium (Hennighausen and Robinson, 1998; Wiesen et al., 1999), although the signaling molecules that mediate the reciprocal interactions are only beginning to be identified.

Epimorphin was originally characterized as a stromal cell surface molecule involved in embryonic epithelial morphogenesis (Hirai et al., 1992); a later study also showed that the same gene encoded a member of the syntaxin family (Pelham, 1993; Bennett et al., 1993). In its capacity as an extracellular morphogen, epimorphin has been shown to control developmental processes in endothelial cells (Oka and Hirai, 1996), liver parenchymal cells (Hirose et al., 1996; Watanabe et al., 1998), embryonic lung cells (Koshida and Hirai, 1997), and pancreatic carcinoma cells (Lehnert et al., 2001). In the mammary gland, epimorphin is present at the surface of both stromal fibroblasts and myoepithelial cells (Hirai et al., 1998), and thus is poised to play a role in mammary morphogenesis. Previously, we used a 3-dimensional collagen assay to characterize the function of epimorphin in normal mammary epithelial cell morphogenesis (Hirai et al., 1998). When presented in a polar, basal fashion, epimorphin produced branching morphogenesis, while apolar presentation triggered formation of structures with large central lumina. The mechanism of its morphogenic activities, however, remained to be identified.

The transcription factor CCAAT/enhancer binding protein β (C/EBPβ) has been implicated in many developmental processes (for review, see Lekstrom-Hines and Xanthopoulos, 1998); however, no upstream effector of C/EBPβ has, as yet, been identified (Robinson et al., 1998). C/EBPβ is found in several isoforms that possess altered transactivation potentials: LAP, the full length 34 kDa isoform, and LIP, a truncated isoform of 20 kDa (Descombes et al, 1991). The relative expression of these isoforms changes throughout development of the mammary gland (Raught et al., 1995). Investigations of mice deficient for expression of C/EBPβ have revealed that this transcription factor is essential for normal mammary gland morphogenesis (Robinson et al., 1998, Seagroves et al., 1998). Among other defects, C/EBP$\beta^{-/-}$ mice were found to have bloated ducts with enlarged central lumina, reminiscent of the luminal structures formed in our culture assays when mammary epithelial cells were presented with epimorphin in an apolar fashion. We hypothesized that this transcription factor might participate in the luminal morphogenesis triggered by apolar presentation of epimorphin to cultured mammary epithelial cells, and that epimorphin may play a similar role *in vivo*. This hypothesis led to several specific predictions. First, presentation of epimorphin to mammary epithelial cells *in vitro* should regulate C/EBPβ, and the morphogenic effects of epimorphin should be reproduced by appropriate artificial regulation of C/EBPβ in cultured mammary epithelial cells. Second, epimorphin should be present at the apical surface of the mammary epithelial cells *in vivo* during the development of alveolar lumina, and apolar presentation of epimorphin to mammary epithelial cells *in vivo* should regulate C/EBPβ and produce enlarged lumina. Here, we present experimental results that confirm these predictions.

Materials and Methods

Cells and tissue culture

SCp2 cells, a normal mammary epithelial cell line (Desprez et al., 1993; Roskelley et al., 1994; Hirai et al., 1998), and primary mammary epithelial cells were maintained in growth medium (DMEM/F12 (Gibco) supplemented with 5% FBS (Hyclone), 5 µg/ml insulin (Sigma), and 50 µg/ml gentamycin). PTSE cells, generated as described in Hirai et al., 1998, were maintained in growth medium supplemented with 5 µg/ml of tetracycline. For generation of SCp2 cells expressing LIP and LAP under control of a tetracycline-repressable promoter, cDNAs were isolated from CMV-LIP and CMV-LAP constructs and were cloned into the *Eco*RI site of the eukaryotic expression vector pTetT-Splice (Life Technologies, Ltd.). SCp2 cells (5×10^5) were transfected with 5µg of this vector, 5µg of pTet.tTAK vector (Life Technologies, Ltd.), and 0.5 µg of pSV40neo (Schmidhauser et al., 1992) using lipofectamine (Life Technologies, Ltd.) according to the manufacturer's instructions. After selection for neomycin-resistant clones in the continuous presence of tetracycline, the expression of LIP and LAP was analyzed by Western blotting in the presence and absence of 5µg/ml tetracycline. SCp2/LIP1, SCp2/LIP2, SCp2/LIP3, and SCp2/LAP cell lines were isolated using this procedure.

Functional assays

Assays for formation of luminal structures were performed essentially as described previously (Hirai et al., 1998). Briefly, cells were grown in serum-free medium either containing 100 µg/ml recombinant soluble epimorphin (for SCp2 cells) or lacking tetracycline (for PTSE cells). In some wells, recombinant epimorphin, H123, was coated prior to seeding the cells (Hirai et al., 1998). The level of protein expression of C/EBPβ gene products was measured by immunoblotting the total cell lysates at day 3, or as described. Morphogenesis assays were performed using clustered cells that were embedded in collagen gels as described previously (Hirai et al., 1998) with slight modifications. Briefly, cell clusters were prepared in agarose gels and suspended in a mixture of 8.5 volumes of collagen type I (Koken, IP) and 1 volume of 10X serum-free medium, adjusted to pH 7.4 with 0.5 volume of alkaline solution. After addition of one volume of PBS, cell clusters were suspended in the collagen solution (50-100 clusters in 100 μl) and poured onto basal collagen gels in individual wells of 48 well plates. Growth medium containing 50 ng/ml EGF and appropriate amount of tetracycline were added to each well. As indicated, soluble recombinant epimorphin was added to a final concentration of 50 μg/ml.

Generation of recombinant epimorphin

All recombinant epimorphin proteins could be solubilized in 1.5 mM HCl, wheras solubility of the proteins was increased by C-terminal truncation. By removal of all of domain 3 (aa 189-264), recombinant epimorphin (termed H12, aa 1-188) is freely soluble in PBS or growth medium. By three-dimensional collagen assay (Hirai et al., 1998), the truncated proteins retained all original morphogenic activities. All recombinant epimorphin polypeptides were produced in *E* coli BL-21 and purified in the presence of 8 M Urea as described previously (Oka and Hirai, 1996) with slight modifications. Deletion mutants of epimorphin tagged with a 6x His sequence were generated by PCR, inserted into the prokaryotic expression vector PET3a (Novagen), and introduced into bacterial cells. All the recombinant proteins purified from the bacteria with Ni-NTA-agarose beads (Qiagen) were dialyzed either directly against 1.5 mM HCl or gradually against 1 M, 0.8 M, 0.5 M, 0.25 M, 0.125 M Urea in ice-cold PBS, followed by urea-free PBS. The soluble fraction in each sample was sterile filtered and assayed for protein concentration.

Antibodies

To prepare antibodies specific for individual domains of epimorphin, antiserum raised against untagged epimorphin (Hirai, 1994) was affinity purified with nitrocellulose membranes precoated with purified recombinant epimorphin fragments (1: aa1-104, 2: aa105-188, 3: aa189-264) as described previously (Hirai et al., 1998). To prepare antibodies recognizing just the C-terminal sequence of epimorphin, affinity purified anti-H123 antibodies were absorbed with a column (mixture of Affigel 10 and 15, Bio-rad) immobilized with histidine-tagged recombinant epimorphin deletion mutant H1-230 (aa 1-230). The antibodies bound to the column were eluted with 0.25 M glycine-HCl (pH 2.7), immediately neutralized with 1M phosphate buffer (pH 8.0), dialyzed against PBS and used as anti-1-230. These antibodies were used for immunoblotting at a concentration of 10 μg/ml. For preparation of the anti-LAPonly antibody, a construct containing the nucleotide sequence of epimorphin from 190-582 was fused with an ATG and a 6x CAT sequence (for 6x His) at the 5' end, was cloned into bacterial expression vector pet 3C', and expressed in *E. coli* strain BL21. The recombinant LAPonly peptide was purified on Ni-NTA gel in the presence of 4M urea, dialyzed against PBS (final purity was greater than 95%), and used for injecting rats. Antibodies to C/EBPα and C/EBPβ gene products were from Santa Cruz Biotechnology (Santa Cruz, CA) and to β-actin was from Sigma (St. Louis, MO). These reagents were used for immunoblotting at the dilution of 1/200. The antibody to T7 peptide (Novagen) was used at 1/1000 dilution.

Analysis of epimorphin cleavage

Lactating mammary gland tissue (1 g) was sonicated in 5 ml of 20 mM Tris-HCl (pH 8.0), 0.5 mM $CaCl_2$, and 25 mM NaCl on ice. After centrifugation at 200 xg for 1 minute, the supernatant was collected and centrifuged at 3000 xg for 30 minutes at 4°C. The pellet was then washed several times with PBS, and re-suspended in 500 μl serum-free medium. A 100 μl portion of this membrane-enriched fraction was mixed with 10 μg of recombinant full-length epimorphin (isoform I) tagged with 6x histidine at the N-terminus (Oka and Hirai, 1996) and incubated at 37°C. After 48 hours, 800 μl of 8M Urea (pH 8.0) was added to the reaction to dissolve all the insoluble materials. Ni-NTA-agarose beads (Qiagen) were then added to the supernatant to collect the His-tagged products. After washing the beads several times with 8 M urea (pH 8.0), the collected products were analyzed by immunoblotting with anti-epimorphin antibodies. Untagged recombinant epimorphin was used as a control. For transfection experiments, the expression vector SRα-296 (Tanabe et al., 1988), containing the full length cDNA for epimorphin isoform I tagged with T7 peptide at the N-terminus (SRαTEPM) (Hirai, 1994), was electrophoretically transfected into SCp2, SCp2', and primary mammary epithelial cells using Bio-rad gene pulser. Cells were incubated in growth medium for 24 hours, then in serum-free DMEM/F12 supplemented with 5 μg/ml insulin (Sigma), 3 μg/ml prolactin (NIH) and 1 μg/ml hydrocortisone (Sigma) for an additional two days. The cDNA products in the cells were analyzed by immunoblotting using an anti-T7 tag monoclonal antibody (Novagen). Supernatants from cultured cells were concentrated by immunoprecipitation using anti-epimorphin antibodies and protein A-Sepharose (Bio-rad) and analyzed by immunoblot.

Estimation of LIP/LAP ratios in cells

Cells were directly dissolved in SDS-sample buffer, electrophoresed in SDS-PAGE gels, and blotted onto PVDF membrane; C/EBPβ gene products were visualized with anti-C/EBPβ antibodies. Nuclear extracts were prepared as described by Deryckere and Gannon (1994). The estimation of intensity of LIP and LAP signals was carried out with ChemiImager 4000 low light imaging system (Alpha Innotech). The relative amount of LIP/LAP was compared to control, which was loaded on the same blot.

Transgenic mice

For generation of WAP-EPM mice, epimorphin cDNA was tagged with a mouse IL-2 signal peptide sequence (5'-ATG TAC AGC ATG CAG CTC GCA TCC TGT GTC ACA TTG ACA CTT GTG CTC CTT GTC AAC AGC GCT CCC-3') generated by PCR as described previously (Hirai et al., 1998). The construct was subcloned into the *Hin*dIII site of the CA10 vector, which contains a whey acidic protein (WAP) gene promoter and a sequence for the 3' untranslated region of WAP protein (Sympson et al., 1994). After linearization with *Not*I, the target gene was purified with glass milk (Bio-rad) and microinjected into fertilized eggs. The injected embryos were then transferred to pseudo-pregnant FVB mice. To determine the presence and integrity of the transgene, DNA was isolated from the tail of offspring of transgenic mice and analyzed by PCR using 5'-AGCACCAGGAGAAGTCAC-3' (from the WAP promoter sequence) as 5' primer and 5'-CGACAGCAGTGTCTGCATCG-3' (from the epimorphin sequence) as 3' primer. For the positive lines, the expression of the transgene was further analyzed by RT-PCR. Briefly, total RNA from pregnant mice was isolated, treated with DNAse, reverse transcribed and analyzed using 5'-ATGCAGCTCGCATCCTCTGTC-3' (from the IL-2 signal peptide sequence) as 5' primer and 5'-CGACAGCAGTGTCTGCATCG-3' (from the epimorphin sequence) as 3' primer.

Results

Apolar presentation of epimorphin regulates LIP

When PTSE mammary epithelial cells, which express epimorphin under control of the tetracycline transactivator, were cultured in the absence of tet for 4 days, they expressed epimorphin in an apolar fashion and formed structures composed of a single cell layer and a central lumen (Fig. 1Aa). Addition of tetracycline at this point repressed the epimorphin transgene and caused the luminal structures to collapse (Fig. 1Ac). However, addition of soluble, recombinant epimorphin (H12) along with tetracycline caused the luminal structures to continue to expand and to develop even larger central lumina (Fig. 1Ab). Using this assay, we found that epimorphin elevated the overall levels of C/EBPβ and increased the relative ratio of the 20 kDa isoform (LIP) to the 34 kDa isoform (LAP) (Fig. 1B). A similar effect was observed in primary mammary epithelial cells (Fig. 1C). Epimorphin also increased the expression of C/EBPα, although the truncated form of this protein was not observed (Fig. 1D), suggesting that the alteration in the LIP/LAP ratio was not due to increased expression of non-specific proteases.

Epimorphin-induced LIP is not produced by proteolysis

To verify that increased LIP did not result from *in vitro* proteolysis, we generated an antiserum against the N-terminal 14 kDa of C/EBPβ, the region contained within LAP that is absent in LIP (Fig. 2A). This antiserum was used to probe a blot containing extracts of control SCp2 cells, SCp2 cells treated with recombinant epimorphin (rEPM), SCp2 cells transiently transfected with LAP (SCp2-LAP cells), or SCp2-LAP cells treated with rEPM (Fig. 2B). We found the expected increase in 34 kDa LAP in both the rEPM-treated samples and in the LAP transfectants, but did not find any 14 kDa band that would have resulted from proteolytic cleavage of LAP. A parallel blot, probed with a commercial antibody directed against a region of C/EBPβ common to LAP and LIP (Fig. 2C), showed a comparable increase in LIP expression in both epimorphin-treated samples. Despite the considerably higher expression of LAP in the SCp2-LAP cells, there was no band corresponding to LIP. A third blot containing extracts from SCp2 cells transiently transfected with either LAP or LIP constructs was probed with the commercial antibody, and no significant degradation of LAP was observed (Fig. 2C). Together, these experiments show that our extraction protocol produces little or no proteolysis of C/EBPβ.

LIP mimics, and LAP blocks the effects of epimorphin

We found that regulation of C/EBPβ isoform expression was sufficient to mediate luminal morphogenesis of cultured mammary epithelial cells using constructs with tetracycline-regulated expression of LIP or LAP (Fig 3A). In collagen assays, untransfected or mock-transfected cells formed aggregates. Moderate expression of LIP, achieved through attenuation of expression in SCp2/LIP1 and SCp2/LIP2 cells and through incomplete repression in SCp2/LIP3 cells, promoted luminal morphogenesis. However, high expression of LIP caused apoptosis, assayed by accumulation of fragmented DNA (data not shown). In a number of experiments, we found that increases in the LIP/LAP ratio between ~2-10 triggered luminal morphogenesis (Figs. 3Bc, 0.5 μg/ml tet; 3Cb), and increases in ratios that were higher than ~10 led to apoptosis (Figs. 3Bb, 0.02 μg/ml tet; 3Cc). By contrast, overexpression of LAP led to formation of compact colonies with no lumina that were resistant to epimorphin-mediated luminal morphogenesis (Fig. 3Dc), possibly through the ability of LAP to neutralize LIP (Descombes et al., 1991; Buck et al., 1994). Taken together, these results demonstrate that epimorphin upregulates the relative expression of LIP to LAP, that constitutive expression of LIP is sufficient to produce luminal morphogenesis, and that constitutive expression of LAP can block epimorphin from producing this morphogenic activity.

Soluble epimorphin is present in milk

In mammary glands from nulliparous animals, we had previously detected epimorphin only on the stromal fibroblasts and myoepithelial cells (Hirai et al., 1998). However, as apolar presentation of epimorphin was important for luminal morphogenesis in culture, we investigated the possibility that endogenous epimorphin might also be present at the apical surface of luminal epithelial cells during normal development. When unfixed lactating glands from wild type mice were stained with a gentle washing protocol, epimorphin could be detected also in the lumina (Fig. 4A). This localization suggested a soluble form of epimorphin, and western analysis of normal mouse milk with anti-epimorphin antibodies identified a ~30 kDa band that was smaller than full-length 34 kDa epimorphin (Fig. 4Bb). The presence of this protein was not due to simple cell lysis, as β-actin, a diagnostic for burst cells (Mather and Keenan, 1988), was not found in the milk (Fig. 4Ba). Western analyses using antibodies specific for domains within epimorphin suggested that the ~30 kDa protein is a C-terminal truncation (Fig. 4C).

We reasoned that the soluble form of epimorphin might result from proteolysis of membrane-bound epimorphin. Consistent with this hypothesis, when full-length recombinant epimorphin containing an N-terminal 6xhis tag was incubated with a membrane extract derived from whole lactating mammary tissue, the protein was cleaved to form a single ~30 kDa product (Fig. 5Bb). Although it was not possible to isolate sufficient quantities of the ~30 kDa species to verify its activity, other C-terminal truncations are fully functional in morphogenesis assays (Hirai et al., 1998).

When an expression construct containing full-length epimorphin with an N-terminal T7 tag was transfected into primary mammary epithelial cells derived from pregnant animals, the cells produced both ~30 kDa and 34 kDa products (Fig. 5Aa). Although the general population of SCp2 cells, when transfected with the expression construct, did not normally produce the soluble ~30 kDa epimorphin species (Fig. 5Ab), a subclone of transfectants, named SCp2', possessed the ability to do so (Fig. 5Ac). Cleavage of tagged epimorphin by SCp2' cell extracts was inhibited by the metalloproteinase (MMP) inhibitor GM6001 (Fig. 5Bb), suggesting that an MMP could mediate the conversion to the soluble ~30 kDa species. The identity of this protease is under investigation.

WAP-epimorphin transgenic mice showed upregulation of LIP

To investigate epimorphin-mediated mammary luminal morphogenesis *in vivo*, we generated transgenic mice that expressed membrane-tethered epimorphin on the surface of luminal epithelial cells. This was accomplished by fusing the IL-2 signal peptide to the epimorphin cDNA and then sandwiching this construct between the promoter and 3' untranslated region of the whey acidic protein (WAP) gene (Pittius et al., 1988; Sympson et al, 1994) to generate the WAP-EPM construct (Fig. 6A). We had previously used a similar construct to localize the epimorphin to the surface of cultured mammary epithelial cells (Hirai et al., 1998).

Two of six founder mice, EP4 and EP6, showed incorporation of the transgene (Fig. 6B). Nulliparous heterozygotes from these lines displayed no detectable increase in epimorphin expression and had no distinctively aberrant phenotype (data not shown). Pregnant and lactating heterozygotes did express epimorphin and also had dramatically enlarged ducts (Fig. 6Cb). In addition, transgenic animals had large and disorganized secretory alveoli. A similar phenotype with varying severity was observed in all of the WAP-EPM mice, but not in any of the transgene-negative littermates examined (n=25), and the phenotype of all the transgenic mice became stronger as animals aged (data not shown). Lactating transgenic mice displaying the strongest phenotype had problems producing milk and would cannibalize their young.

When analyzed by western blot, mammary glands from WAP-EPM mice showed a dramatic increase in C/EBPβ relative to wild-type mice, both in total protein and in the relative ratio of LIP to LAP (Fig. 6D). Thus, the WAP-EPM mice paralleled the culture studies, as apolar expression of epimorphin on mammary epithelial cells upregulated the LIP/LAP ratio and led to enlarged ductal lumina.

Discussion

In this study, we have identified a functional relationship between epimorphin-triggered luminal morphogenesis and increased relative expression of the LIP isoform of C/EBPβ. This was both necessary and sufficient for the morphogenic activity in cultured cells, as expression of LIP induced luminal morphogenesis, while expression of LAP blocked luminal morphogenesis induced by epimorphin. Since apical presentation of epimorphin seemed to be important for this morphogenic process, we examined epimorphin expression *in vivo* and found a truncated, soluble form present in mouse milk. The WAP-EPM transgenic mice supported the role of epimorphin as effector of LIP-mediated luminal morphogenesis. These animals express epimorphin in an apolar fashion on luminal epithelial cells and also have increased LIP/LAP ratios and greatly enlarged ductal lumina in pregnant and lactating animals.

It is unclear if epimorphin-induced luminal morphogenesis is caused by a positive activity of LIP or by the downregulation of LAP by LIP. The latter possibility is supported by the similarities between mammary ductal morphology of WAP-EPM and C/EBPβ$^{-/-}$ mice. It should be noted, however, that the phenotype of the WAP-EPM mice is significantly different from the phenotype of the C/EBPβ$^{-/-}$ mice. The latter have severe ovarian dysfunction and consequent female sterility (Sterneck et al., 1997), as well as defective differentiation of myeloid cells (Screpanti et al., 1995; Tanaka et al., 1995), adipocytes (Tanaka et al., 1997), hepatocytes (Lee et al., 1997), and keratinocytes (Zhu et al., 1999), and aberrant expression of lactogenic hormone receptors (Seagroves et al., 2000). Even when comparisons are limited to the mammary gland, there are still substantial differences, as lobuloalveolar development was inhibited in the C/EBPβ$^{-/-}$ mice (Seagroves et al., 1998). These differences may be partially attributed to the spatio-temporal modulation of epimorphin expression by the WAP promoter, and the consequent limitation of the affected cell population. Furthermore, differences in mouse strain backgrounds may also be relevant.

Soluble epimorphin in murine ductal lumina

Examination of normal mouse milk revealed a soluble molecule of ~30 kDa that was cross-reactive with all antibodies to epimorphin except those generated against the C-terminal sequence. The hypothesis that the truncated epimorphin could result from proteolytic cleavage at the cell surface was examined by reconstitution experiments in which membrane fractions from mammary glands of lactating mice were incubated with tagged recombinant epimorphin. We found that this treatment resulted in selective generation of the ~30 kDa form (Fig. 6B). A number of other signaling molecules, including the *kit* ligand and TGFβ, are released by membrane-bound MMPs (Blobel, 1997; Werb, 1997), and we found that a specific inhibitor of MMPs inhibited the conversion of full-length epimorphin to the ~30 kDa form in SCp2' cells.

It is unclear how soluble, extracellular epimorphin, if produced by stromal cells, is transported to the ductal lumina. Possibly, after removal of the membrane-anchoring sequence by proteolysis, epimorphin could be delivered by transcytosis across the epithelial membrane, or it could pass through temporary junctions between luminal epithelial cells. Such mechanisms are involved in the delivery of other stromal proteins to the luminal space (Linzell and Peaker, 1973; Grosvenor et al., 1992; Stelwagen et al., 1997; Ollivier-Bousquet, 1998). Alternatively, as some mammary epithelial cell lines are able to express epimorphin in culture (Hirai et al., 1998), it is possible that a subpopulation of luminal epithelial cells produce soluble epimorphin during development. In this case, secretion of the large volume of milk proteins and lipid droplets by the lactating gland (Burgoyne and Duncan, 1998; Mather and Keenan, 1998) may act as a vehicle for efficient distribution of epimorphin. Currently, there are no data to distinguish between these possibilities.

Regulation of the LIP/LAP ratio

LAP and LIP are mutually antagonistic isoforms of C/EBPβ (Descombes and Schibler, 1991; Buck et al., 1994). In our culture system, moderate expression of LIP led to lumen formation while expression of LAP blocked this process. That high expression of LIP produced apoptosis is an intriguing observation in light of the role of apoptosis in the formation of lumina in terminal endbuds and alveolar structure in culture (Humphreys et al., 1996). The possibility that LIP may contribute to this process warrants careful analysis in 3-D cultures and *in vivo*. However, as morphogenic processes are likely to be orchestrated within microdomains of the mammary gland, specific antibodies and quantitative, real-time imaging may be required.

LIP has been implicated in other physiological processes as diverse as inflammation (An et al., 1996), liver regeneration (Timchenko et al., 1998; Welm et al., 2000), development (Diehl et al., 1994; Darlington, 1999), and aging (Hseih et al., 1998; Timchenko et al., 1998). LIP was originally proposed to arise through alternative translation by leaky ribosome scanning (Descombes and Schibler, 1991; Ossipow et al., 1993). Subsequent investigations implicated a small, out of frame ORF (uORF) in the 5' sequence of C/EBPβ as a regulator of LIP/LAP ratios and determinant of cell differentiation (Calkhoven et al., 2000, and references therein). Some studies have suggested that a specific RNA-binding protein may control the alternative translation initiation (Timchenko et al., 1999; Welm et al., 2000). Other investigations have shown that LIP can result from a mechanism that is regulated by C/EBPα (Welm et al., 1999), and in this regard, it is relevant that apolar presentation of epimorphin leads to upregulation of C/EBPα, as well (Fig. 1D). In other cases, observation of the LIP isoform may be due to *in vitro* proteolysis of LAP (Lincoln et al., 1998; Baer et al., 1998; Baer and Johnson, 2000), but we excluded this possibility in our system (Fig. 2).

Epimorphin is a member of the syntaxin family

The epimorphin gene also encodes syntaxin-2, shown to function during targeting/fusion of intracellular vesicles with the plasma membrane (Bennett et al., 1993; Pelham, 1993). Although syntaxins have been shown to act at the cytoplasmic face of membranes (Brose, 1993; Pelham, 1993, Rothman, 1994; Wheeler et al., 1996; Jagadish et al., 1997), the subcellular localization of syntaxins can be modulated through various mechanisms (Hui et al., 1997; Jagadish et al., 1997; Rodger et al., 1998; Quiñones et al., 1999), and syntaxins have been found at the cell surface, at least transiently (Smirnova et al., 1993a, b; Hirai et al., 1993, 1998; Butt et al., 1996; Nagamatsu et al., 1997; Guo et al., 1998). Evidently, these complex and versatile proteins can perform multiple functions, and it will be a considerable challenge to discover how these diverse roles are related. However, functional relationships of known protein motifs within epimorphin/syntaxin-2 may provide some clues. The SNARE motif is a highly conserved domain within the syntaxin family that is located between aa 188-253 of epimorphin (Weimbs et al., 1997). Although domain analyses of syntaxins demonstrate the SNARE motif is essential for formation of competent membrane fusion complexes (Sutton et al., 1998; Jahn and Südhof, 1999), we have found that this motif is not required for the morphogenic activity of epimorphin in mammary epithelial cells, since a genetically engineered epimorphin (H12) lacking the SNARE motif is fully competent in our assays (Figure 1 and Hirai et al., 1998). These findings support a model in which syntaxin/epimorphin molecules harbor distinct functional domains that allow the molecules to function in more than one cellular context.

The mechanism by which epimorphin is translocated to the outside of the plasma membrane is not known, although cell surface localization of other membrane-associated molecules lacking signal peptides has been reported (Wen et al., 1992; Skach et al., 1993; Ostapchuk et al., 1994; Guo et al., 1998). In such cases, the membrane topology depends upon the three-dimensional conformation of the N-terminal sequence (Denzer et al., 1995; Spiess, 1995; Wahlberg and Spiess, 1997). Similarly, intramolecular interactions within the epimorphin sequence (Hirai, 1994) may allow a subpopulation to orient itself on the outer surface of the plasma membrane.

In conclusion, we have shown that epimorphin can mediate luminal morphogenesis of mammary epithelial cells *in vitro* by control of C/EBPβ, and we have implicated this mechanism in mammary morphogenesis. The conclusive link will require further analyses of WAP-EPM, WAP-LIP, and WAP-LAP transgenic mice, generated in the same strain background, as well as a knockout of C/EBPβ in our assay system. Through such experiments, it would be possible to determine if C/EBPβ is the only mediator of epimorphin-triggered luminal morphogenesis *in vivo*. Characterization of the molecular mechanism by which epimorphin controls C/EBPβ isoform levels will require analysis of the epithelial receptor for extracellular epimorphin; it should be easier to identify this receptor by using the soluble form of epimorphin, identified in this study, as a probe. These investigations are currently underway.

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Figure Legends

Figure 1. Effects of apolar presentation of epimorphin. (A) Epimorphin activates luminal morphogenesis. PTSE cell clusters were cultured in collagen for four days in the absence of tetracycline (EPM ON) (a), then cultured for an additional four days in the presence of tetracycline (EPM OFF) and with (b) or without (c) H12 form of epimorphin in the medium. The luminal diameter of more than 20 clusters in each category was measured; graph bar indicates standard deviation. Scale bar: 100 μm. (B) Epimorphin increases C/EBPβ expression and the LIP/LAP ratio. PTSE cell clusters were cultured on plastic in the presence or absence of tetracycline, and SCp2 cells were cultured on recombinant full-length epimorphin (rEPM) or collagen (Hirai et al., 1998), or in the presence of rEPM/H12. Analyses of unclustered SCp2 cells incubated on tissue culture plastic or plastic coated with collagen are shown as controls. CRM, cross-reactive material (Seagroves et al., 1998). Estimated LIP/LAP ratios, relative to the controls, are indicated. (C) LIP and LAP are dramatically upregulated by both epimorphin transfection (Sig EPM) and addition of rEPM to primary mammary epithelial cells. The identity of the band between CRM and LAP is unknown. (D) SCp2 cells cultured in the presence of rEPM also upregulate C/EBPα. For (B), (C), and (D), the results are typical of three independent experiments.

Figure 2. Minimal proteolysis of LAP occurs during sample preparation. (A) Diagram depicting the targeted location of the anti-LAPonly antibody and of the commercial anti-C/EBPβ antibody. (B) Western blot probed with the anti-LAPonly antibody. (C) Western blots probed with commercial anti-C/EBPβ antibody. *Left*: parallel blot to B; asterisk, cross-reactive material. *Right:* blot of SCp2 cells, transiently transfected with LAP or LIP expression plasmids. Results shown are typical of two independent experiments.

Figure 3. Characterization of clones that conditionally express LIP and LAP. (A) Analysis of C/EBPβ gene products in LIP and LAP transfectants, cultured in the presence (5 μg/ml) or absence of tetracycline (tet). Results shown are typical of three independent experiments. B & C - Behavior of LIP-transfected cells cultured in various concentrations of tetracycline. Clusters of SCp2 controls (Ba) and of SCp2/LIP1 and SCp2/LIP2 in 0.5

μg/ml tet (Bb) formed compact colonies in collagen (shown in Ca for SCp2/LIP1, 0.5 μg/ml tet). SCp2/LIP3 expresses moderate levels of LIP and forms lumina in the presence of tet (Bc, Cb). Reduction of tet in medium of SCp2/LIP1 and SCp2/LIP2 cells results in apoptotic cell death (Bb, shown in Cc for SCp2/LIP1, 0.02 μg/ml tet). Bar 100μm. (D) Induction of LAP transgene inhibits luminal morphogenesis. (a) LAP expression in clustered parental SCp2 cells. (b, c) Clustered parental Scp2 cells and SCp2/LAP cells, cultured in collagen gels in the absence of tetracycline (tet) and in the presence and absence of rEPM. For B, C, and D, at least twenty colonies from each condition were examined.

Figure 4. Identification of ~30 kDa soluble epimorphin *in vivo*. (A) Frozen sections of prefixed (a) and unfixed (b) lactating mammary glands labeled with anti-epimorphin antibodies. The tissue in (b) was fixed on the slide immediately after sectioning, and the staining was carried out with mild washing so as not to remove soluble proteins in the lumina (asterisk). Bar: 30 μm. (B) Immunoblot analysis of the lactating mammary gland tissue (T) and milk (M) with anti-β-actin and anti-epimorphin antibodies. 5 μg (x5) or 1 μg protein samples of mammary gland extract (T) or milk (M), collected from lactating wild type mice, were probed with anti-β-actin (a) and epimorphin (b) antibodies. (C) 30 kDa soluble epimorphin reacts with all anti-epimorphin antibodies except those directed against the C-terminus. (a) Schematic diagram of affinity purified antibodies targeted to different domains of epimorphin. (b) Immunoblot analyses of milk and recombinant full length 34 kDa epimorphin (r-EPM) using the affinity-purified antibodies. Anti-1, -2, -3, anti-1-230 and anti-c are specific to epimorphin domains aa1-104, 105-188, 189-264, 1-230, and 231-264, respectively.

Figure 5. Production of ~30 kDa soluble epimorphin *in vitro*. (A) The products of epimorphin cDNA tagged with T7 peptide at the N-terminus in transfected primary mammary cells (a), SCp2 cells (b), and SCp2' cells (c), were analyzed by immunoblot with monoclonal anti-T7 antibody. Cells (C) and supernatants (S) were analyzed separately. For detection in the supernatant, an immunocomplex with anti-epimorphin antibodies was collected with protein A-sepharose beads. Untagged recombinant epimorphin was used as

a control. (B) Recombinant epimorphin tagged with 6x his at the N-terminus was incubated with membranes derived from lactating mammary glands (a) or from either SCp2 or SCp2' cells (b). Products were collected with a Ni-agarose column and analyzed by immunoblotting with anti-epimorphin antibodies. For (a), untagged recombinant epimorphin was used as a control. In (b), the MMP inhibitor GM6001 or the inactive structural homologue C1004 were added to a final concentration of 10µM. Results are typical of three independent experiments.

Figure 6. WAP-EPM transgenic mice have enlarged ductal lumina and altered C/EBPβ expression. (A) Schematic diagram of the transgene construct. N; *Not*I, H; *Hin*dIII. (B) PCR analysis of the integration of the transgene into genomic DNA and RT-PCR analysis of epimorphin transgene expression. As a control for RT-PCR, endogenous stromelysin-1 (SL-1) was analyzed in the same samples. Lower bands (asterisk) are unreacted primers that remained in control samples after RT-PCR. (C) Phenotypic appearance of the mammary gland (circled) from mid-pregnant (7 day) normal (a) and transgenic (b) mice. Bar: 5 mm. Whole-mount stained mammary gland of normal (c) and transgenic (d) mice. Bar: 300µm. (D) Analysis of C/EBPβ gene products in mammary tissue of normal and transgenic mice using commercial anti-C/EBPβ antibody. CRM, cross-reactive material.

Figure 1

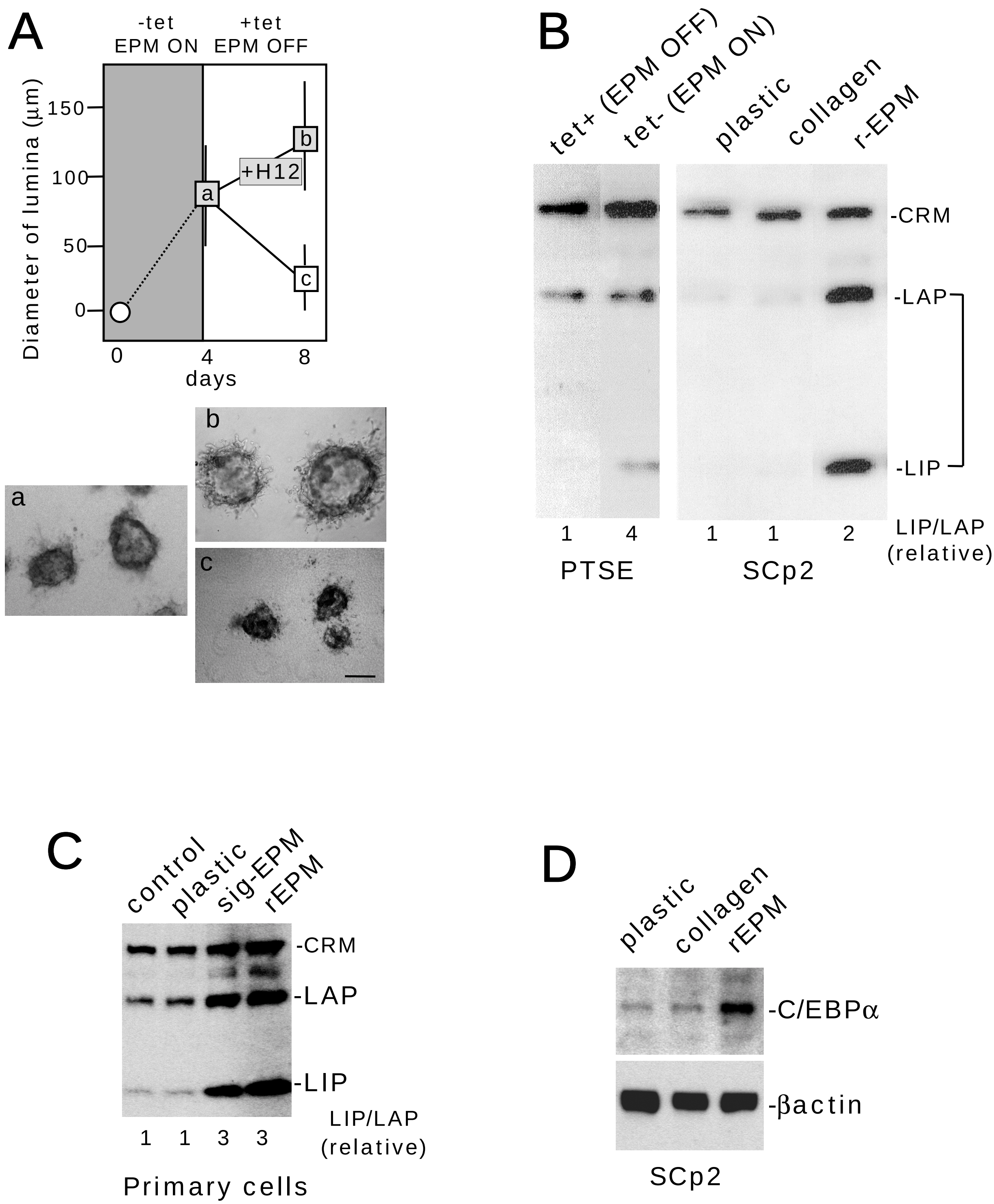

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Figure 2

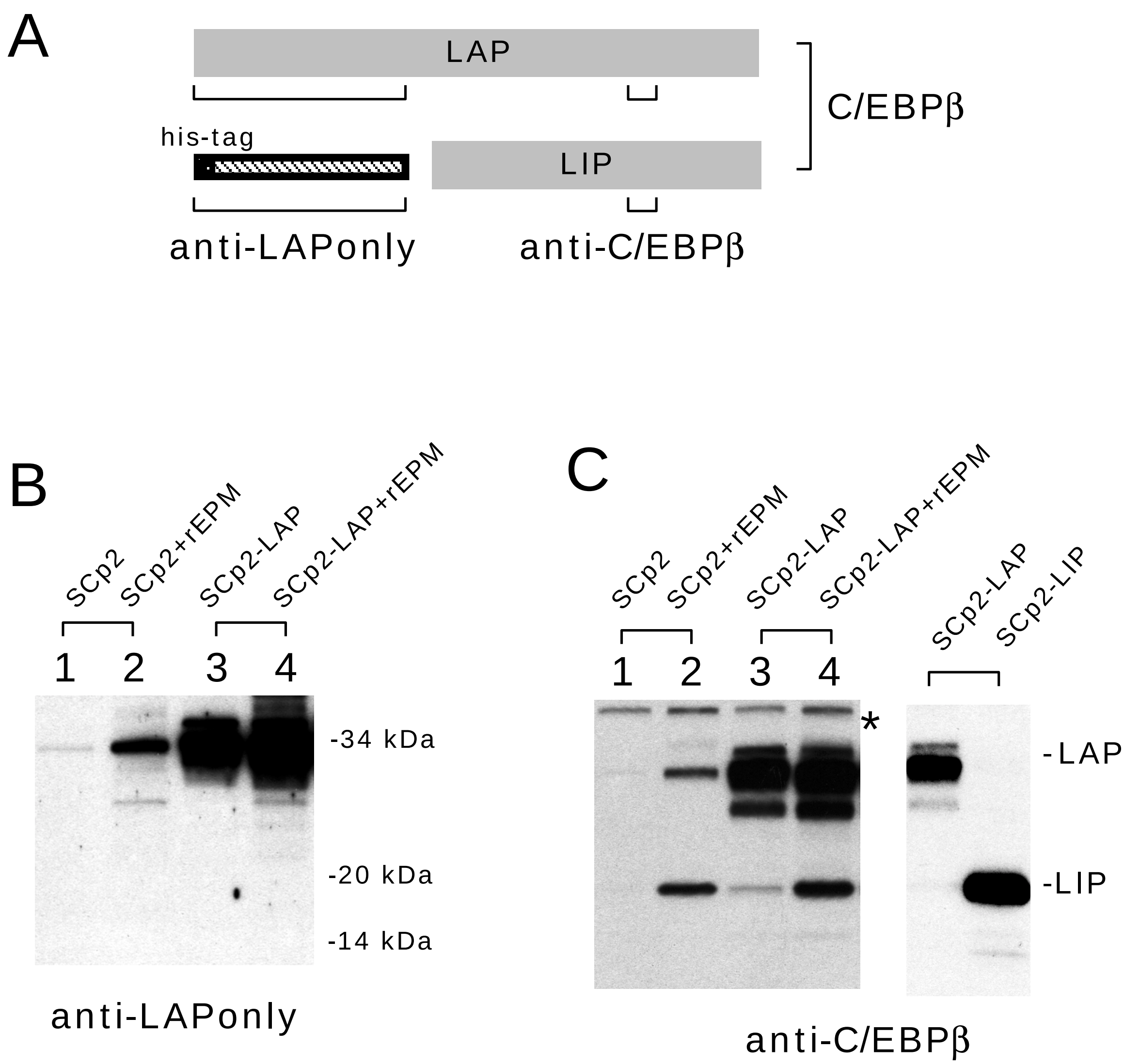
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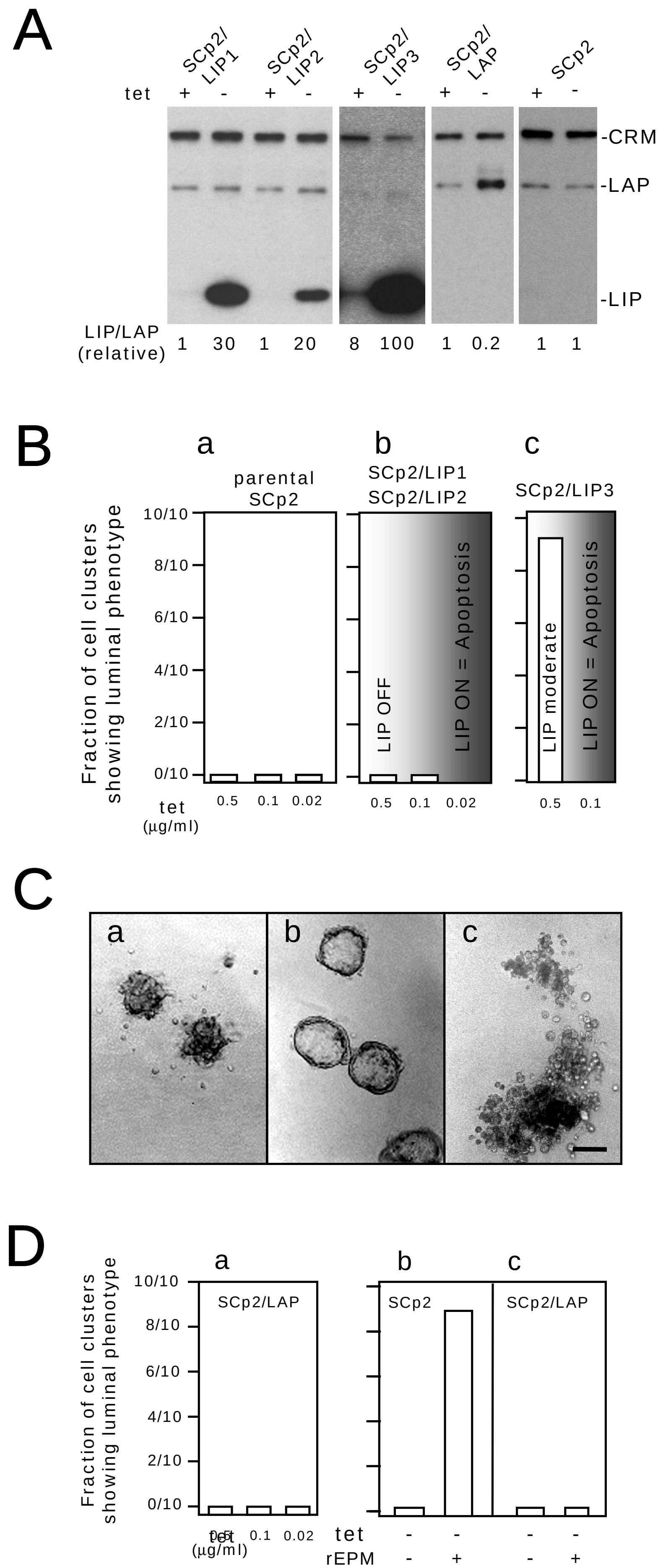
Figure 3



A
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-LIP
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SCp2/LIP1
SCp2/LIP2
c
SCp2/LIP3
Fraction of cell clusters showing luminal phenotype
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8/10
6/10
4/10
2/10
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LIP OFF
LIP ON = Apoptosis
LIP moderate
LIP ON = Apoptosis
tet (µg/ml)
0.5 0.1 0.02
0.5 0.1 0.02
0.5 0.1
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Fraction of cell clusters showing luminal phenotype
10/10
8/10
6/10
4/10
2/10
0/10
SCp2/LAP
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Figure 4

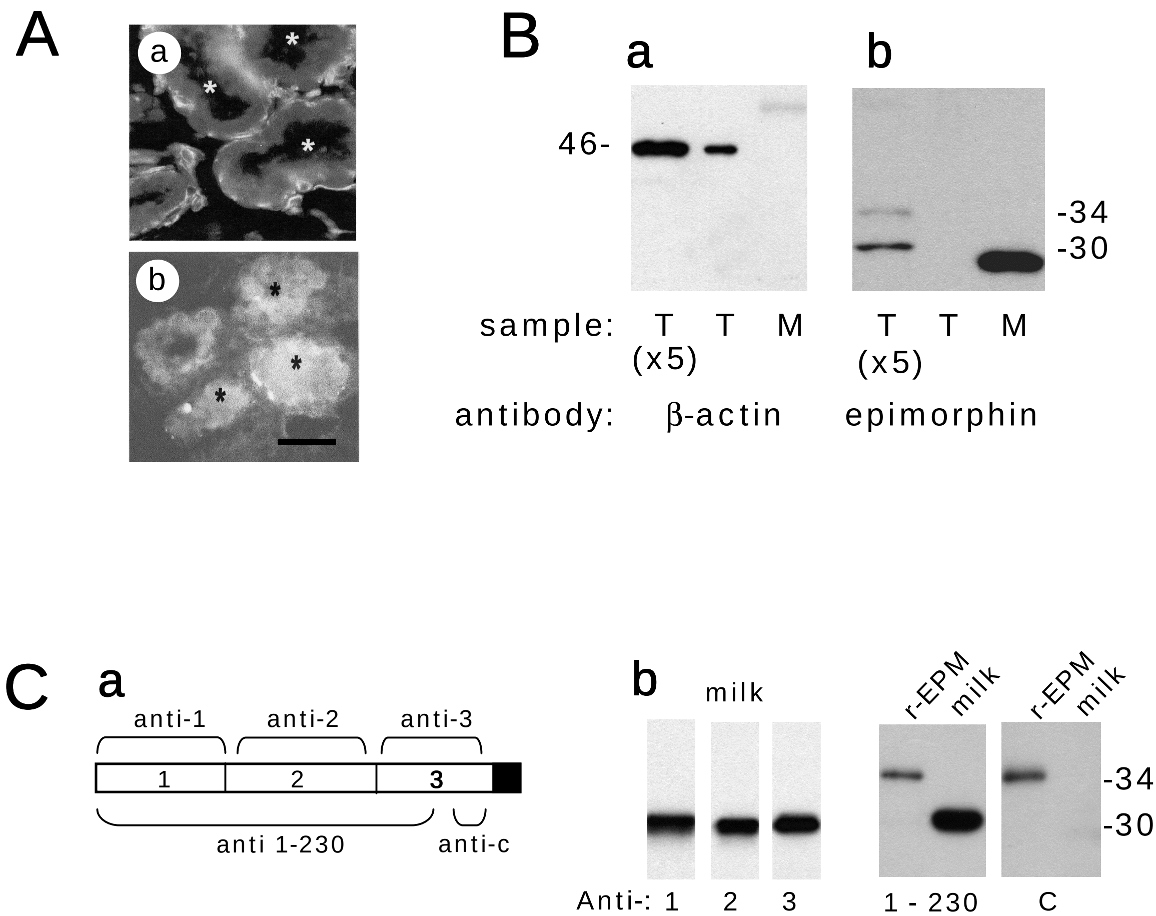
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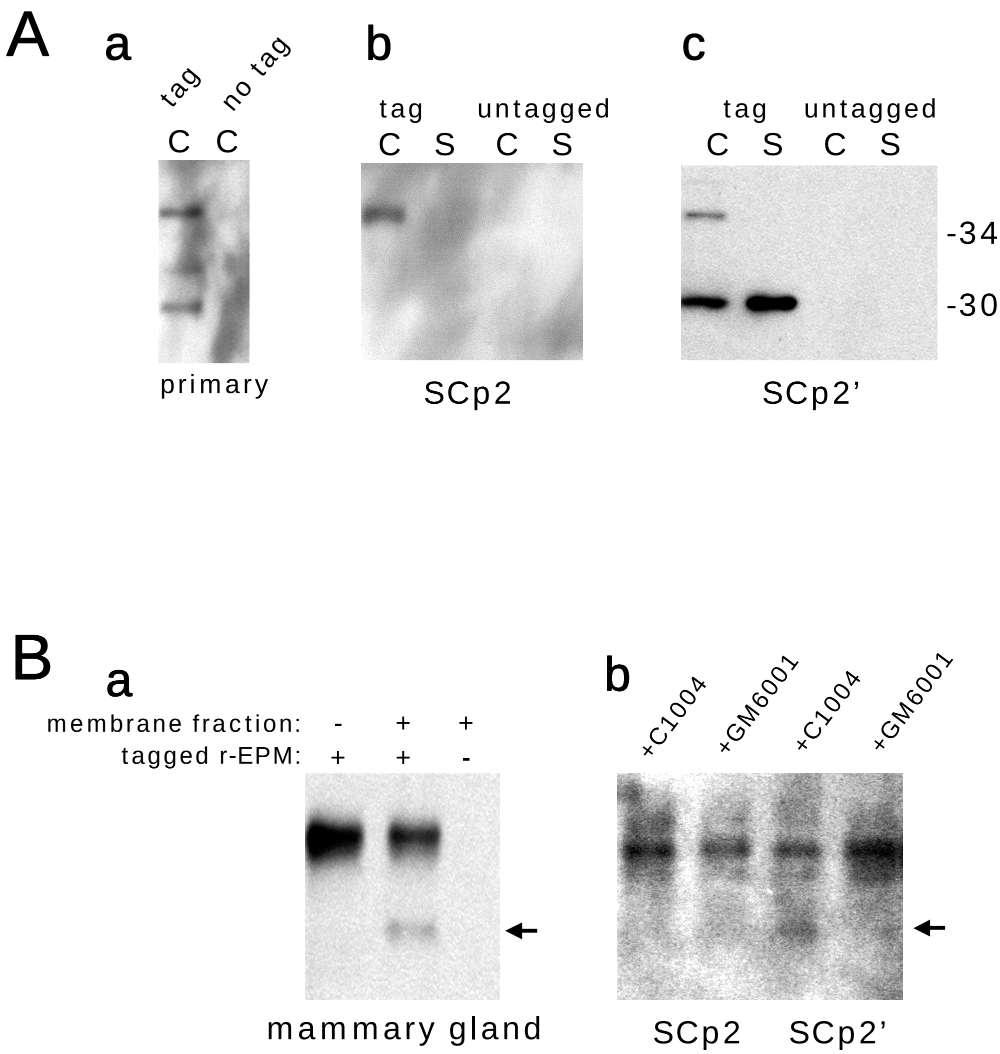
Figure 5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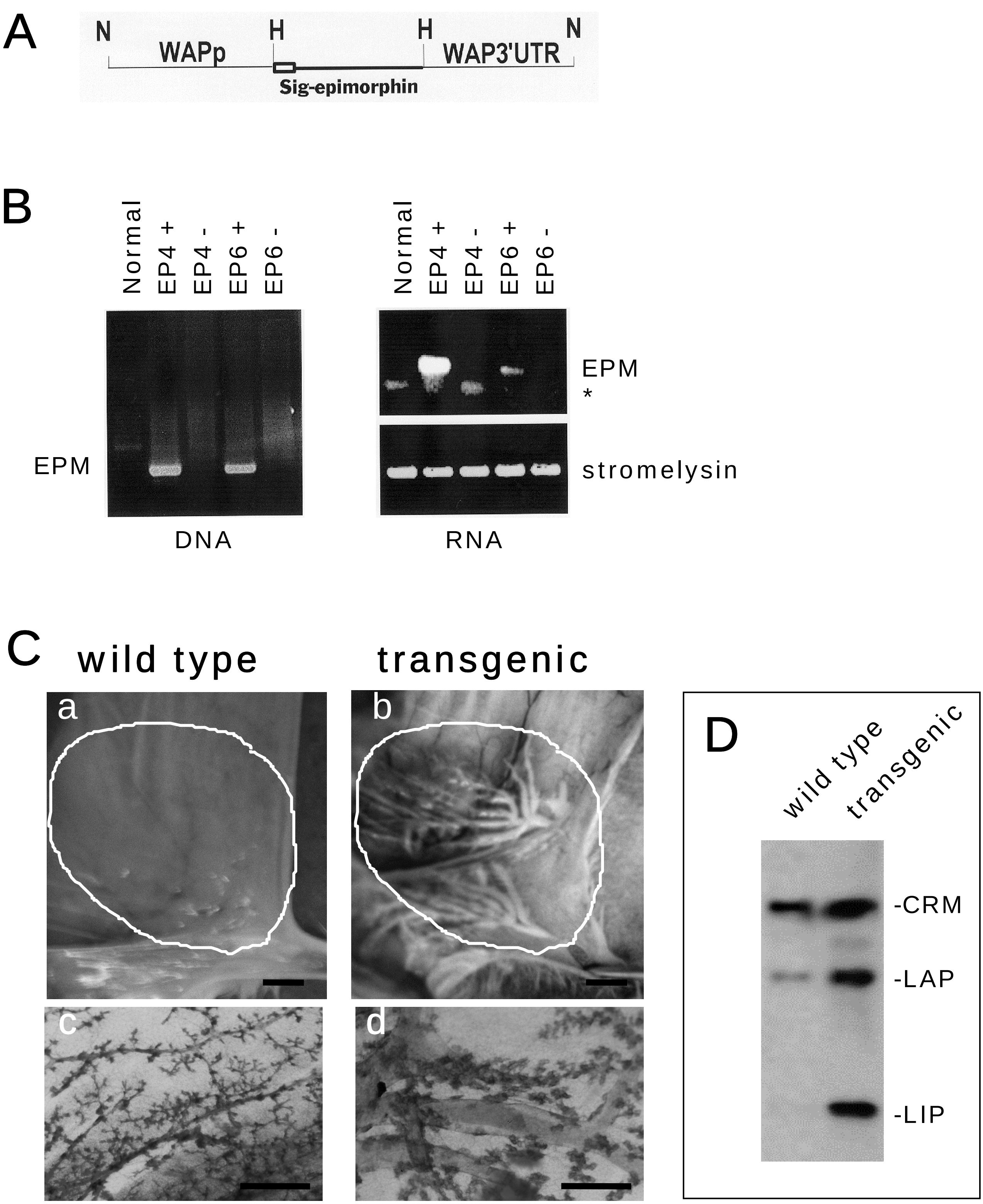
a
membrane fraction: - + +
tagged r-EPM: + + -
mammary gland
b
+C1004
+GM6001
+C1004
+GM6001
SCp2 SCp2'

Figure 6


A
N
WAPp
H
H
WAP3'UTR
N
Sig-epimorphin
B
Normal
EP4 +
EP4 -
EP6 +
EP6 -
EPM
DNA
Normal
EP4 +
EP4 -
EP6 +
EP6 -
EPM
*
stromelysin
RNA
C
wild type
transgenic
a
b
c
d
D
wild type
transgenic
-CRM
-LAP
-LIP